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Protein, Vitellogenin, and Vitellin Levels in the Hemolymph and Ovaries during Ovarian Development in *Penaeus semisulcatus* (de Haan)

S. SHAFIR[1,2], M. TOM[1], M. OVADIA[2], AND E. LUBZENS[1]

[1]*National Institute of Oceanography, Israel Oceanographic & Limnological Research, P.O. Box 8030, Haifa 31080, Israel, and* [2]*Dept. of Zoology, Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

Abstract. The concentration of vitellogenin (Vg) in the hemolymph of *Penaeus semisulcatus* was found to increase from an average of 50 μg ml^{-1} to 439 μg ml^{-1} in female shrimp during ovarian development. The most significant increase in Vg occurred concomitant with the increase in the vitellin (Vt) content of oocytes with an average diameter (AOD) ranging between 150 and 250 μm. The amount of Vt in the oocytes was found to increase linearly from a mean of 0.0126 mg to 4.55 mg per gm body weight. However, the percentage of Vt in the total protein was found to decrease, from 67% in ovaries with AOD of 150–250 μm, to 39.7% in ovaries with AOD of 350 μm or larger. The volume of the hemolymph was found to be 0.4 ml per gm body weight and did not change significantly during ovarian development. Assuming that Vg in the hemolymph represents either an extraovarian origin of Vt or an active secretion from the ovary, a turnover rate of two to three times per day was calculated over one full cycle of oocyte development. However, during the most significant increase in Vt in the ovary (in ovaries with AOD of 150–250 μm), the turnover rate in the hemolymph could reach seven to eight times per day. The results lead to the conclusion that the contribution of Vg to the formation of the ovary is quantitatively insignificant.

Introduction

Vitellogenesis is associated with several complex processes that lead to the formation of mature oocytes within the ovary. In crustaceans, substantial quantities of yolk accumulate within the developing oocytes, and serve to meet the basic requirements of embryonic and larval development, independent of the maternal organism (Adiyodi and Subramoniam, 1983). One of the major components of yolk is the lipoglycoprotein, vitellin (Vt). In several crustacean species, Vt was found to be synthesized by the ovary (Lui *et al.*, 1974; Lui and O'Connor, 1976, 1977; Dehn *et al.*, 1983; Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989a, b; Browdy *et al.*, 1990). Yano and Chinzei (1987) suggested that Vt was synthesized in the follicle cells of *Penaeus japonicus*. However, a protein that reacts immunologically to the antiserum prepared against purified Vt was detected in the hemolymph of vitellogenic females. This female-specific protein (FSP), known as vitellogenin (Vg), has been reported in all species studied so far (Kerr, 1969; Ceccaldi, 1970; Fyffe and O'Connor, 1973; Wolin *et al.*, 1973; Caubere *et al.*, 1976; Meusy, 1980; Dehn *et al.*, 1983; Ferrero *et al.*, 1983; Marzari *et al.*, 1986; Susuki, 1987; Tom *et al.*, 1987; Nelson *et al.*, 1988; Quackenbush and Keeley, 1988; Quackenbush, 1989a). Vg is one of the two lipoproteins known in crustacean hemolymph (Lee, 1990). A large increase in the concentration of Vg has been reported in the hemolymph during vitellogenesis. While some reports show that the appearance of Vg in the hemolymph is correlated with morphological changes in the ovaries antecedent to egg release, others show that Vg is at the highest concentrations prior to the maximum accumulation of yolk in the oocyte (Quackenbush, 1989b; Lee, 1990).

Several reports show evidence for extraovarian tissues engaged in Vg synthesis. Vg was reported to be produced

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Abbreviations: AOD—Average oocyte diameter; SAT—Subepidermal adipose tissue; Vg—Vitellogenin; Vt—Vitellin; HEP—Hepatopancreas.

by the subepidermal adipose tissue (SAT) of anostracean (Van Beek *et al.*, 1987), isopod (Picaud, 1980; Souty and Picaud, 1981; Picaud *et al.*, 1989) and amphipod crustaceans (Croisille and Junera, 1980; Junera and Croisille, 1980). Vg was also found in the SAT of decapods (Meusy *et al.*, 1983; Vazquez-Boucard, 1985; Tom *et al.*, 1987). However, it was not identified as being synthesized by *in vitro* incubated SAT of *P. semisulcatus* (Fainzilber, 1988; Fainzilber *et al.*, 1992). Conflicting results were reported for Vg synthesis by the hepatopancreas (HEP) in decapods. Quackenbush and Keeley (1988) and Quackenbush (1989a, b) reported *in vitro* Vg synthesis in *Uca pugilator* and *P. vannamei*, respectively. However, Yano and Chinzei (1987) and Rankin *et al.* (1989) were unable to detect *de novo* Vg synthesis in HEP of *P. japonicus* and *P. vannamei*, respectively. Low levels of a Vt-immunoreactive protein were detected in *in vitro* incubated HEP in females of *P. semisulcatus* (Fainzilber, 1988; Fainzilber *et al.*, 1992). The possible contribution of Vg in the accumulation of Vt in the ovary is further suggested by work describing endocytosis in the ovary (Hinsch and Cone, 1969; Wolin *et al.*, 1973; Duronslet *et al.*, 1975; Zerbib, 1979; Beams and Kessel, 1980; Schade and Shivers, 1980; Zerbib and Mustel, 1984; Jugan and Soyez, 1985). Furthermore, Vg receptors were isolated from the ovarian cytoplasmic membranes of two decapod species (Jugan, 1985; Lavrdure and Soyez, 1988; Jugan and Van Herp, 1989). However, Lui and O'Connor (1976) suggested that Vg originated from resorbed oocytes.

The present investigation aims at elucidating the possible role of Vg and its contribution to oocyte development in *Penaeus semisulcatus*, which is a species of economic importance in Israel. We attempted to examine the hypothesis that Vg in the hemolymph is transported and taken up by the ovary, relying on the suggestion of Byard and Aiken (1984). They reported that the observed levels of FSP in the hemolymph were always higher prior to the maximum accumulation of yolk in the oocytes, and that they dropped off markedly prior to oviposition. The conclusion drawn by Byard and Aiken was that this pattern is consistent with the idea that the FSP represents an externally synthesized protein and is found at the highest level in hemolymph during the period when oocytes are accumulating the maximum amount of yolk. To examine this possibility, the concentration of Vg in the hemolymph, Vt in the ovary, and the volume of the hemolymph were determined during one cycle of ovarian maturation. The results enabled us to calculate the hypothetical turnover rate assuming that the entire Vt within the ovary originated from Vg circulating in the hemolymph.

An approach to vitellogenesis as a flow system, in which Vg is produced by an extraovarian tissue, released into the hemolymph, and subsequently taken up by the developing oocytes, was described in insects (Bakker-Grunwald and Applebaum, 1977). This approach was also attempted in the present paper, in order to reach conclusions on the possible dynamic flow of Vg in the hemolymph.

Materials and Methods

Chemicals

^{3}H Inulin (46/mCi/g) was purchased from New England Nuclear (U.S.A.). All other reagents were of analytical grade and were purchased from Sigma (U.S.A.), Merck (Germany) or Bio-Rad (U.S.A.).

Animals

Adult *P. semisulcatus* were collected in Haifa Bay, Israel. They were held in 3 m^3 running seawater tanks, at a density of 30 specimens per tank. Water was changed at a rate of 300% per day. Salinity was constant at 40‰, and temperature ranged from 18°C (winter) to 27°C (summer). The animals were fed once a day on a mixture of frozen fish, squid, shrimp and *Artemia*. Females were individually marked by clipping of the uropods, and ovarian development was monitored externally, according to the methods of Browdy and Samocha (1985). Moulting of marked females was also recorded by identifying the uropod coded cuts of the shedded exuviae.

Results of hemolymph volume, protein and Vg levels were related to the average oocyte diameter (AOD), measured according to Shlagman *et al.* (1986) in sacrificed animals.

Collection of hemolymph and the determination of its volume

Hemolymph was collected from animals bled after cutting off the anterior part of the cephalothorax near the base of the eyestalks. The hemolymph from each female was collected into a vial containing a known volume of 10% sodium citrate solution.

The hemolymph volume was determined by injecting 2 μCi of ^{3}H-inulin dissolved in 100 μl of sterilized seawater diluted to 30 ppt. Preliminary experimental results showed that the hemolymph radioactivity was stable for 24 h after the injection of the inulin. The hemolymph volume was calculated by measuring the dilution in the radioactivity of samples withdrawn 2 h after the injection of the labeled inulin.

Determination of proteins, Vg, and Vt concentrations

The total protein concentration in the hemolymph and ovaries was determined according to Bradford (1976), using bovine serum albumin as a standard.

The concentrations of Vg in the hemolymph samples and Vt in the ovarian homogenates were determined by

rocket immunoelectrophoresis (Jurd, 1981). The area formed by the "rocket" precipitation lines was calculated using a Complot series digitizer 7000 and compared to concentrations of purified Vt subjected to the same "rocket" preparation. The purified Vt and the rabbit polyclonal antibody used in these determinations were isolated and prepared as described in Browdy *et al.* (1990).

Experimental procedure

Two series of experiments were carried out. In Experiment 1, hemolymph samples were removed (as described previously) from 77 females that were at various stages of ovarian development, including females immediately after the moult. The hemolymph samples from each female were centrifuged (9000 × *g* at 4°C for 10 min in a Sorvall RC-5C U.S.A.) and the supernatant was stored at −70°C. In parallel, a small piece was removed from the ovaries of each female and fixed in formaldehyde (4% in seawater) for the measurements of AOD (see above). In these females, the protein and Vg concentration in the hemolymph were determined (see above) and related to the AOD.

In Experiment 2, 21 females were chosen individually, after monitoring for several days the moult and ovarian developmental stages. Females following moulting, those showing arrested ovarian development, and those with ovaries in progress of oosorption were rejected. Each female was injected with ^{3}H inulin (as described above). After 2 h, the females were weighed and bled and the hemolymph volume was determined as described above. Ovaries were dissected out and weighed. A small piece was fixed in formaldehyde and used for measurements of AOD. Two other samples (each weighing 0.1–0.2 g) were used for determinations of dry weight. They were placed in pre-weighed vials and dried at 60°C until reaching constant weight (about 48 h). The remaining part of the ovary was homogenized in 10 ml of phosphate buffer (0.1 *M*, pH 7.4) and centrifuged at 4°C and 9000 × *g* for 20 min (Sorvall RC-5C, U.S.A.). The supernatant of the ovarian homogenates and the hemolymph samples were stored at −70°C, until they were used in the determinations of total protein, Vt, and Vg concentrations.

Results

The concentrations of total protein and Vt specific protein (Vg) in the hemolymph was found to increase with the progress of ovarian development, shown by the increase in AOD (Table 1). The values obtained in Experiment 1 for protein, Vg and Vg of 77 females and 73 females, respectively, were pooled and divided into four size groups according to their AOD. It can be clearly seen that females with AOD of 150 μm or larger had similar

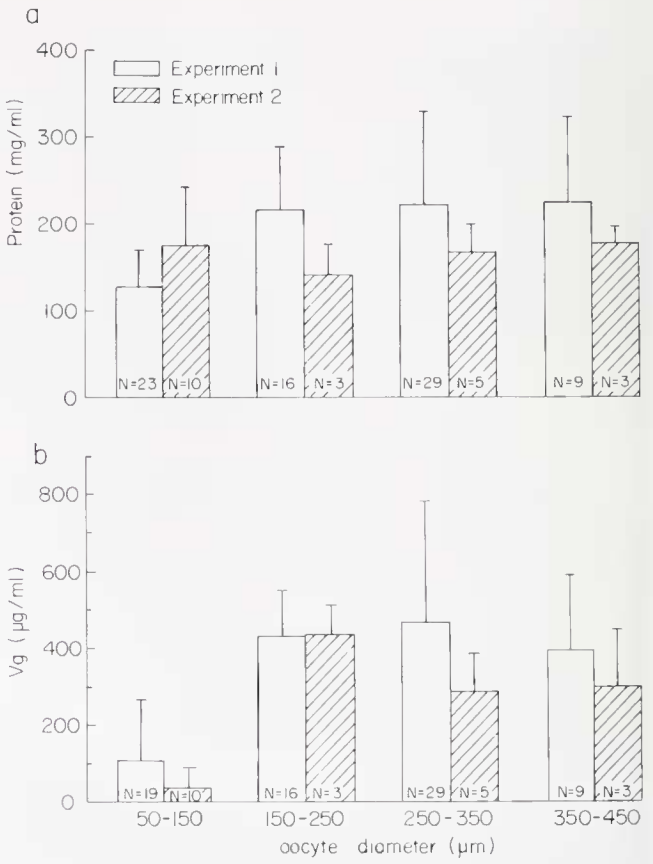

Figure 1. The concentration of (a) protein in mg ml^{-1} and (b) vitellogenin in μg ml^{-1} in the hemolymph (Experiments 1 and 2) related to the average oocyte diameter (AOD). The mean ± S.D. is shown for samples pooled into four size groups of AOD.

levels of total protein and Vg (one way ANOVA, $P > 0.05$). However, females at the initial stage of oocyte development (50–150 μm in AOD) show lower protein levels ($P < 0.001$) and Vg levels ($P < 0.0001$). Four of the examined females which were taken immediately after spawning showed an average of 430.0 ± 43.8 μg ml^{-1} of Vg in their hemolymph, and their results were not included in Fig. 1b. The highest average values of protein (247 ± 139 mg m^{-1}) and Vg (562 ± 410 μg ml^{-1}) were found in females with an AOD ranging between 250 and 300 μm. The lowest average values for protein (116 ± 32 mg ml^{-1}) and Vg (60 ± 95 μg ml^{-1}) were found in females with an AOD of less than 100 μm. The results from the 21 females (Experiment 2) were divided into 4 size groups according to the AOD of the ovaries (Fig. 1a, b). Some of the females included in the size class of 50–150 μm AOD were postmoult. This may have contributed to the large standard deviations in this group, relative to the others. Unlike the results shown earlier for Experiment 1, the total protein concentration in these females did not change with the AOD. However, Vg was virtually not

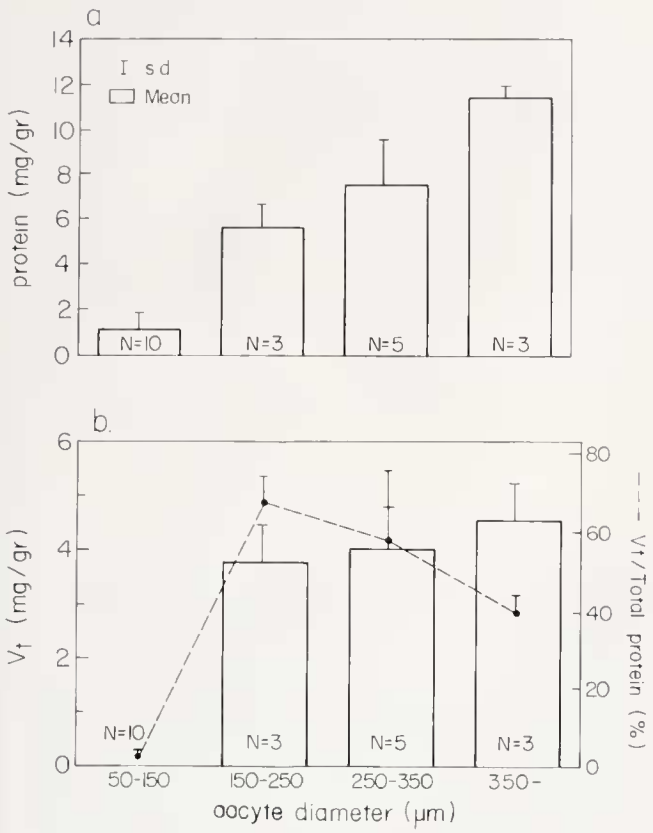

Figure 2. The total amount of protein (a) and vitellin (b) in mg/g fresh weight and the percent of vitellin out of the total amount of protein in ovaries of females at various stages of ovarian development. Results (mean ± S.D.) were pooled into four size groups according to the average oocyte diameter.

present in the hemolymph samples removed from females with an AOD of 50–150 μm.

The mean concentrations of Vg in the hemolymph in the four oocyte size groups of Experiment 1 were not statistically different from those of Experiment 2 (P ranging from 0.2 to 0.9). However, the mean concentration of protein in the hemolymph in females from Experiment 1 with oocytes ranging in size from 50–150 μm was significantly lower ($P < 0.02$) than that of females from the same size group in Experiment 2. These differences may be due to the fact that in Experiment 1 the 50–150 μm size group included post-moult females and post oviposition females, which were eliminated in Experiment 2.

The hemolymph volume did not change in relation to the AOD of the examined females (results not shown) and its average was 0.403 ± 0.114 ml per g body weight.

The total amount of protein in the ovary (in Experiment 2) increased linearly from an average of 1.14 ± 0.7 mg per g body weight to 11.4 ± 0.4 mg per g body weight (Fig. 2a). The amount of Vt in the ovaries increased significantly in ovaries with AOD of 150–250 μm and only slightly in ovaries with larger AOD (Fig. 2b). The Vt concentration in the ovaries was found to average 4.55 ± 0.66 mg per g body weight in ovaries with an AOD of 300 μm or larger. A significant increase in the relative amount of Vt in the total ovarian proteins was found when the AOD increased from 50–150 μm to 150–250 μm, similar to that found in the hemolymph. This ratio decreased significantly towards the end of ovarian development (Fig. 2b). The water content in the ovary was found to decrease linearly during oocyte maturation (Fig. 3), from 83% in immature females to 69.5% in mature ones. This could partially explain the increase in the total amount of protein in the ovaries mentioned earlier (Fig. 2a).

Discussion

The concentration of Vg in the hemolymph of *P. semisulcatus* was found to range from 50 μg ml^{-1} in females with undeveloped ovaries to a high average concentration of 439 μg ml^{-1} in females with oocytes larger than 150 μm AOD. These values constitute only 0.06 ± 0.09% to 0.27 ± 0.15% of the total proteins in the hemolymph of females with undeveloped or developed ovaries, respectively. The relatively large standard deviations probably stem from the attempt to correlate Vg level to the AOD in preset size classes. These values are much lower than those reported for *P. vannamei* (Quackenbush, 1989a), for *P. japonicus* (20 μg μl^{-1} at the maximum stage in vitellogenesis; Yano, 1987), for *Homarus americanus* (1 mg ml^{-1}; Nelson *et al.*, 1988), for *Cancer antennarius* (estimated as 175 mg dl^{-1}; Fig. 1 in Spaziani, 1988), for *Callinectes sapidus* (4.1 mg ml^{-1}; Lee and Puppione, 1988), or for *Macrobrachium rosenbergii* (12 mg ml^{-1}; Derelle *et al.*, 1986). Most of the protein in the hemolymph of several species of crustaceans was found to be associated with hemocyanin (Magnum, 1983; Depledge and Bjerregaard, 1989).

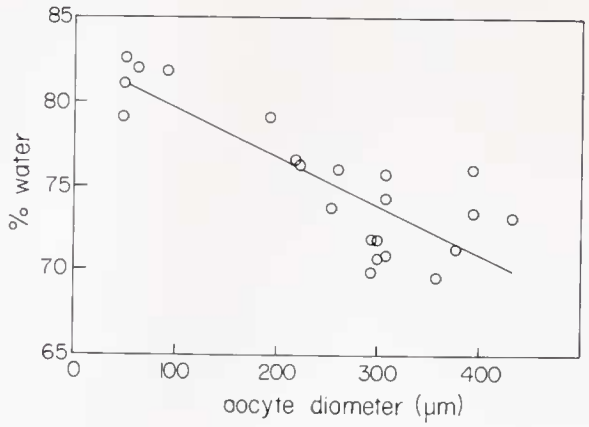

Figure 3. The percent of water in ovaries related to the average oocyte diameter (R2 = 0.814).

Table I

The calculated turnover rate of Vg in the hemolymph at four different stages of ovarian development, using the mean values of the hemolymph volume, Vg concentration, and the amount of Vt in the ovaries, at each stage

Average oocyte diameter (AOD; μm)	n	Hemolymph volume (ml per g body weight)	Vg in hemolymph (μg per g body weight)	Total amount of Vg in ovaries (mg per g body weight)	Increment in the amount of Vt in ovaries (mg per g body weight)	Turnover rate* (per day)	Turnover rate** (per day)
50–150	10	0.5317	1.145	0.0126	0.0126	0.16	0.16
150–250	3	0.4361	8.83	3.76	3.745	7.80	7.78
250–350	5	0.3573	5.63	4.03	0.2726	16.02	1.08
350–450	4	0.4720	7.40	5.11	1.0785	11.69	2.47

* Calculated from the total amount of Vt in ovaries.

** Calculated from the increment in the amount of Vt in ovaries.

The concentration of total protein was found to increase (in Experiment 1) in the hemolymph with the increase in oocyte size, staying at a stable level at later stages of oocyte development. However, no such trend was found in Experiment 2. It is possible that in Experiment 1, the lower concentrations could be attributed to females that were taken immediately after moulting, when their AOD was less than 100 μm. The hemolymph volume was reported to increase prior to moulting, and an inverse linear relationship between hemolymph volume and protein concentration was reported by Smith and Dall (1982).

As mentioned, the concentration of Vg in the hemolymph was found to increase dramatically during the initial stages of oocyte development, when the AOD was 150–250 μm. This increase occurred with a concomitant increase in the absolute and relative amount of Vt in the ovaries. A similar relationship between the increase of Vg in the hemolymph and oocyte development was reported by Lee and Puppione (1988) for *Callinectes sapidus*. However, in *Homarus americanus*, the increased Vg levels in the hemolymph preceded ovarian development (Byard and Aiken, 1984). In ovaries with AOD of 150–250 μm, the Vt constituted 67.7 ± 6.4% of the total proteins. This was reduced to an average of 39.7 ± 4.5%, suggesting that, in addition to Vt, other proteins (*e.g.*, the cortical crypts protein, see below) were synthesized in the ovary towards the end of ovarian maturation. In *P. vannamei*, Vt was found to constitute 5.3–20.9% of the total protein in spawns of fertile eggs (Quackenbush, 1989b).

The presence and synthesis of a cortical crypts protein was reported by Bradfield *et al.* (1989) to take place mainly at the period immediately before spawning in *P. vannamei*. Ovaries that were incubated *in vitro* were also found to synthesize 40–60% of the total proteins. Furthermore, the proportion of Vt was reduced in ovaries with AOD of 350 μm and larger (Browdy *et al.*, 1990). The increase in the total amount of protein deposited in the ovaries was inversely related to their water content, which was reduced from about 83% to 69.5% in the fully mature ovaries.

A flow system demonstrating the Vg flow in hemolymph towards the ovary in vitellogenic females was demonstrated in insects (Bakker-Grunwald and Applebaum, 1977). This model was based on the following data: (a) concentration of Vg and Vt in the hemolymph and ovary, respectively; (b) the rate of incorporation of injected labeled amino acid into Vg; and (c) the rate of incorporation of Vg by the ovary. In this paper, we present results in *P. semisulcatus* that are comparable to (a) above.

In crustaceans, the origin of Vg in the hemolymph is still problematic. In the case that Vg originates from extraovarian tissue, it could be assumed to be directed towards the ovary, where it is taken up and constitutes at least part of the Vt. This is the case in some crustacean species and in most insects which are also arthropods (Kanost *et al.*, 1990). However, several reports show that Vt is synthesized in the ovary (see references in the Introduction). In this case, Vg originates either by controlled secretion from the ovary (Yano and Chinzei, 1987), or by passive leakage from oocytes in the process of resorption. If Vg is actively secreted from the ovary, it could have a role in the transport of nutrients (*e.g.*, lipids) into the ovary from extraovarian tissues (*e.g.*, HEP). The data presented in the present paper allow us to consider the flow dynamics of such systems. From the total hemolymph volume, and the Vg and Vt concentrations in the hemolymph and ovaries, respectively, it is possible to calculate the hypothetical turnover rate of Vg in the hemolymph. This is presented in Table I. If it is assumed that the entire amount of Vt present in the ovaries is transported via the hemolymph as Vg, the calculated turnover rate is 2.5 times per day over one full cycle. In carrying out this calculation, it is assumed that one full cycle of oocyte development is linear and takes 10 days

(Browdy, 1988). However, if the full cycle is divided into four stages, the turnover rate must reach 7.78 per day, to account for the Vt accumulated in oocytes of 150–250 μm. The accumulation rate is reduced at later stages (Table 1), to account for the increment in the amount of Vt accumulated in the ovaries.

The results presented here, on the increase in Vg concentration concomitant with the increase in the amount of Vt in the ovary, and those of Fainzilber *et al.* (1992) show that if there is an extraovarian source for Vt in *P. semisulcatus*, its contribution to Vt is quantitatively very low.

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